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GGDEF-EAL domain proteins in bacterial physiology

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Abstract

GGDEF and EAL domain proteins were identified in the chromosome of many bacteria. GGDEF domain proteins are generally diguanyl cyclases which synthesize the secondary messenger cyclic di-GMP. EAL domain proteins are generally c-di-GMP specific phosphodiesterase which degrade c-di-GMP. C-di-GMP is involved in the regulation of multiple phenotypes in bacteria like biofilm formation, motility and virulence. In this work, we found that c-di-GMP inversely regulates biofilm formation and motility in bacteria such as *Salmonella enterica* serovar Typhimurium, *Escherichia coli* and *Pseudomonas aeruginosa*. Thereby, GGDEF domain proteins up-regulate c-di-GMP concentrations and the biofilm phenotype, while down regulating the motility phenotype. EAL domain proteins have the opposite effect. Consequently, cyclic di-GMP controls the transition from sessility to motility. Phenotypic convergence is also observed when GGDEF domain proteins are exchanged between different species. Mutual complementation of GGDEF domain proteins mutants of *S.* Typhimurium and *Yersinia pestis* restored the respective phenotypes, cellulose biosynthesis and biofilm formation. In S. Typhimurium, the rdar morphotype, a multcellular behaviour that involves biofilm formation is regulated by c-di-GMP. The rdar morphotype is characterized by the expression of extracellular matrix components cellulose and curli fimbriae. CsgD is a major transcriptional regulator required for the activation of biosynthesis of cellulose and curli fimbriae. Systematic analysis of the 5 GGDEF, 7 EAL and 7 GGDEF-EAL domain proteins encoded on the S. Typhimurium chromosome indicates that these proteins do have overlapping, but never a redundant function. Mutant analysis revealed that the GGDEF-EAL domain proteins STM2123 and STM3388 enhance the expression of the transcriptional regulator CsgD at the transcriptional and post-transcriptional level and, consequently, rdar morphotype development, although no change in intracellular c-di-GMP levels is observed. Chromosomal inactivation of AdrA does not have any significant effect on CsgD expression, although AdrA contributes to 60% of the basal level of c-di-GMP at a specific time point. On the other hand, four EAL or GGDEF-EAL domain proteins down-regulate the CsgD concentrations in the cell. While the c-di-GMP degraded by STM1703 is highly dedicated to regulate CsgD expression, c-di-GMP degraded by STM4264, STM3611 and STM1827 is only partially used to down-regulate CsgD expression. C-di-GMP metabolism is connected with other pathways in the cell like degradation of mRNAs. The expression of the EAL domain protein STM4264 dedicated to regulate CsgD expression and STM3611 dedicated to regulate motility are inversely regulated by exoribonuclease poly-nucleotide phosphorylase (PNPase), so that CsgD expression and motility are down-regulated. This finding explains the coherent regulation of opposing phenotypes by PNPase.

List of Publications

This thesis is based on the following papers, which are referred to in the text by their roman numerals

I. Simm, R., Morr, M., **Kader, A.,** Nimtz., and Römling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di GMP levels and transition from sessility to motility. *Mol Microbiol* **53**: 1123-1134

II. Simm, R., Fetherston, J. D., **Kader, A.,** Römling, U., and Perry, R. D. (2005) Phenotypic convergence mediated by GGDEF-domain- containing proteins. *J Bacteriol* **187**: 6816-6823

III. **Kader, A.,** Simm, R., Gerstel, U., Morr, M., and Römling, U. (2006) Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **60:** 602-616

IV. Simm, R., Lusch, A., **Kader, A.,** Andersson, M., and Römling, U. (2007) Role of EAL- containing proteins in multicellular behaviour of *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **189**: 3613-3623

V. **Kader, A.,** Römling, U., and Rhen, M., (2007) Polynucleotide phosphorylase promotes biofiolm formation and motility through differential regulation of EAL domain proteins in *Salmonella enterica* serovar Typhimurium. Manuscript

Contents

List of abbreviations

AdrA	AgfD regulated protein A
BapA	Biofilm-associated protein A
Bcs	Bacterial cellulose synthesis
bdar	brown_dry and rough
C. crescentus	*Caulobacter crescentus*
C-di-GMP	Cyclic diguanosine monophosphate
CR	Congo Red
Csg	Curli subunit gene
CSP	Cold Shock Protein
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia coli*
EPS	Exopolysaccharide
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
G. xylinus	*Gluconacetobacter xylinus*
mRNA	messenger ribonucleic acid
NTS	Non Typhoidal Salmonella
P. aeruginosa	*Pseudomonas aeruginosa*
pdar	pink_dry and rough
PNPase	Polyneucleotide phosphorylase
rdar	red_dry and rough
RPH	RNase PH
saw	smooth and white
SPI	Salmonella Pathogenicity Island
SPV	Salmonella plasmid virulence
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
T3SS	Type 3 Secretion Systems
V. cholerae	*Vibrio cholerae*
X. campestris	*Xanthomonas campestris*
Y. pestis	*Yesinia pestis*

1.0 Introduction

The social activities and organization of diverse groups of bacteria are immensely important to their ecological success in different niches. Most of the surfaces on this planet are teeming with microorganisms which create ecosystems. Bacteria can form biofilm on any surface with moisture and nutrient (Kolter and Greenberg, 2006). Studies on these societies revealed that bacteria can be either in planktonic or in biofilm state. Bacterial communities are widely recognized as being important for survival and persistence in harsh environments (Johnson, 2007). Bacteria in complex communities are using small molecules, which are recognized as a general mechanism, in signal transduction integrating internal and external information to elicit appropriate responses. Cyclic diguanyl monophosphate (c-di-GMP) is a small molecule that has a great impact in the transition from bacterial single cell to biofilm state (Romling *et al.*, 2005; Simm *et al.*, 2004). Mass genome sequencing identified that free living bacteria harbour multiple copies of GGDEF and EAL domain proteins (Galperin *et al.*, 2001). GGDEF and EAL domain proteins are synthesizing and degrading c-di-GMP respectively. Historically, researchers have studied bacterial signalling as if it functioned as a set of isolated, linear pathways. Current studies have demonstrated that many signalling pathways interact and many of the pathways should be construed as intricate networks (Pruss *et al.*, 2006). C-di-GMP and polynucleotide phosphorylase (PNPase) regulation in bacteria have some common attributes which have significant impact on biofilm formation (Kader *et al.*, 2006; Simm *et al.*, 2004), virulence gene expression (Clements *et al.*, 2002).

The infection caused by Salmonella is likely to continue causing significant morbidity and mortality on a global scale. Bacterial biofilm formation has both beneficial and detrimental effects. The roles of GGDEF-EAL domain proteins in signal transduction and regulation of biofilm helps us to understand the biofilm biology. This thesis describes the functions and different phenotypes regulated by the novel second messenger molecule c-di-GMP. It also deals with newly discovered targets for c-di-GMP. Further more, the connection between c-di-GMP signaling and PNPase regulation is the topic of this thesis.

1.1 *Salmonella enterica* serovar Typhimurium as a model organism to study GGDEF and EAL domain proteins

The genus *Salmonella* comprises Gram negative flagellated rods which are belonging to the family *Enterobacteriacea*. The genus name honors the American microbiologist D. E. Salmon. The genus is divided into seven subspecies and more than 2500 serological variants generally termed as serovars. *Salmonella enterica* serovar Typhimurium (S. Typhimurium) has become a model system to study basic biology.

As a model organism to study the functions and task distribution of GGDEF and EAL domain proteins, we choose S. Typhimurium. We focused on the virulent isolate ATCC14028 in order to mine the wealth of information available for S. Typhimurium. S. Typhimurium also has a manageable numbers of GGDEF and EAL domain proteins (Galperin *et al.*, 2001; Kader *et al.*, 2006; Romling and Amikam, 2006; Simm *et al.*, 2007).

1.2 The epidemiology of Salmonellosis

The *Salmonella* infection is immensely important because it causes virulent infections world wide. *Salmonella* is causing an estimated 3 billion human infections per year while the World

Health Organization (WHO) estimates, typhoid fever increased up to 22 million cases. The global burden of the disease estimates 200000 deaths in a year (Crump *et al.*, 2004). In developing countries, the enteric fevers are transmitted by the feco-oral route, through contaminated water and food which are associated with substandard sanitation and hygiene. The epidemiology of the paratyphoid fever is less well described compared to typhoid fever. It is estimated that approximately 25% of enteric fever may be caused by *Salmonella paratyphi* (*S. paratyphi*) (Crump *et al.*, 2004). The number of infections caused by *S. Paratyphi* A has increased in South Asia (Ochiai *et al.*, 2005) and cause infection as severe as *S. typhi* (Maskey *et al.*, 2006). It is difficult to mine the data concerning the non-typhoidal *Salmonella* (NTS) infection. In most cases, patients do not need to consult with physicians. In European Union figures approach 350000 reported cases of NTS infection annually with approximately 1000 deaths (Fisher, 2004a, b).

S. Typhimurium infection is a major cause of gastroenteritis with many thousands of cases reported each year in European Union. S. Typhimurium is associated with food borne infections involving raw milk, cheese, chicken and pigs (Foley *et al.*, 2007; Parveen *et al.*, 2007). S. Typhimurium can cause infection when spread through person to person contact (Steere *et al.*, 1975). It exploits inflammation to compete with the intestinal microbiota. In animal studies, it was shown that oral infection of mice with S. Typhimurium causes meningitis and infection to the brain (Wickham *et al.*, 2007). Meningitis caused by S. Typhimurium is not common in adult but occurs in children (Kauffman and St Hilaire, 1979; Swe *et al.*, 2008). S. Typhimurium was isolated from cerebrospinal fluids and blood culture from HIV positive man (Swe *et al.*, 2008). At a global level, *Salmonella* infections continue to have a large impact on human and animal health.

1.3 Antibiotic resistance in *Salmonella*

Bacteria frequently encounter different selective pressure which results in emergence of resistance. Salmonella is developing resistance at an alarming rate and options for antibacterial treatments are becoming limited. S. Typhimurium is developing antibiotic multi-drug resistant. S. Typhimurium isolated from neonatal meningitis showed to be resistant against penicillin, aminoglycosides, chloramphenicol and cephalosporin (Vahaboglu *et al.*, 1995). Plasmids were isolated from antibiotic resistant S. Typhimurium (Chiu *et al.*, 2002; Vahaboglu *et al.*, 1995). Antibiotic resistant S. Typhimurium was isolated from food poultry samples (Parveen *et al.*, 2007). Studies on different multidrug resistant bacteria were assessed for biofilm formation. It was shown that there is inter-strains variation among the multidrug resistant bacterial strains to form biofilm (Kim and Wei, 2007). Fluoroquinolone treatment can not be used as a treatment regimen for *S. Typhi* and *S. Paratyphi* A infection (Threlfall *et al.*, 2006). Class I integrons have been described in *S Typhi* and these mobile elements are capable of acquiring different antibiotic resistance cassettes. Generally the antibiotic resistance is acquired in Salmonella either by horizontal gene transfers for acquisition of ready-made resistance or mutation of an existing allele. Alarmingly, isolates of *S. Typhi* and *S. paratyphi* A from humans are reported to exhibit decreased susceptibility to ciprofloxacin (Dutta *et al.*, 2001; Saha *et al.*, 2006; Threlfall *et al.*, 2006)

2.0 Biofilm- a multicellular behaviour of bacteria

2.1 Bacterial biofilm formation

Biofilms are assemblies of microorganisms which are closely associated with different surfaces and enclosed in self produced matrices. Numerous biofilm matrix materials have been identified such as polysaccharides, nucleic acids and proteins (Branda *et al.*, 2005; Olsen *et al.*, 1989; White *et al.*, 2001; White *et al.*, 2003; Zogaj *et al.*, 2001). Microbial biofilm is formed on any surfaces with moisture and nutrients. The surfaces can be biotic or abiotic in nature. The broad definition of biofilm states that it is matrix enclosed bacterial populations that adhere to each other on surfaces or interfaces (Costerton *et al.*, 1995). Bacteria in biofilm phenotype are closely associated with each other and they are remarkably resistant to selective pressure, for example antibiotic treatment. Bacteria form biofilm for multiple purposes. Bacterial communities are present in our natural environment as well as in persistence infection (Hancock, 2001). Bacteria produce different biofilm matrix components based on the conditions and the surfaces. The expression of biofilms are greatly affected by environmental conditions (Gerstel and Romling, 2001; Gerstel *et al.*, 2003). The ecological success of a bacterial community is highly dependent on the formation of biofilm matrix (Kolter and Greenberg, 2006). Bacteria release certain compounds and communicate with other cells with these compounds through a mechanism called quorum sensing. If the quorum sensing molecules come to a thresh-hold level that leads to the activation of genes that control a variety of functions including biofilm formation.

The phenotypic switch of bacteria from single cell state to biofilm state is a complex and tightly regulated process. The regulatory networks are controlled by different regulatory genes in a cascade. The complex 3D structure of biofilm shows a heterogeneous population of bacteria and microenvironment within the structure (Fux *et al.*, 2005). The expression of different biofilm matrix also varies with the habitat of the organism, while the same biofilm matrix can be expressed in different environments (Romling *et al.*, 2003; Zogaj *et al.*, 2003). The bacteria can produce a number of biofilm matrices (Branda *et al.*, 2005). The presence of different subpopulations within the biofilm could be due to the different environmental stress and conditions encountered in different microenvironments within the biofilm (Boles and McCarter, 2002; Branda *et al.*, 2005; Stoodley *et al.*, 2002).

2.2 Significance of bacterial biofilm

Bacterial biofilm formation shows different roles in different niches and environment. Biofilm formation can be advantageous. Biofilm forming bacteria can be more resistant to antibiotic treatment. It was shown in *Pseudomonas aeruginosa* (*P. aeruginosa*) that sub-inhibitory concentration of aminoglycoside antibiotics can induce biofilm formation (Hoffman *et al.*, 2005). Biofilm forming bacteria can not be efficiently killed by disinfectants (Scher *et al.*, 2005). Bacteria which are growing in a biofilm state can cause persistence infection (Johnson, 2007). Colonization of bacteria to establish persistent infection in immuno-compromised patients is initiated by biofilm forming bacteria. Bacteria forming biofilm can attach on many surfaces including catheters which show a great risk of re-infection. The host and infectious bacteria are in competition for essential metal since iron is strictly limited in urinary tract (Hancock *et al.*, 2008). Availability of iron greatly influenced the biofilm formation by strains that cause urinary tract infection. Diverse groups of bacteria from the oral

cavity also form biofilm. *Streptococcus mutans*, a member of the dental plaque community has been shown to be involved in the carious process (Welin-Neilands and Svensater, 2007).

Microbial fuel cells (MFCs) convert biodegradable materials into electricity contributing to an array of renewable energy. There are now known microorganisms that can metabolize cellulose and transfer electrons to solid extracellular substrate. The conversion of cellulosic biomass to electricity requires a syntrophic microbial community that uses an insoluble electron donor (cellulose) and electron acceptor. Electricity was generated from cellulose in a MFCs using a defined co-cultures of the cellulolytic fermenter *Clostridium cellulolyticum* and the electrochemically active *Geobacter sulfurreducens* (Ren *et al.*, 2007). Biofilm can increase the surface tension and helps in efficient electron transfer.

Bacterial biofilm formation has an impact on mining. It is associated with secondary gold grains (Reith *et al.*, 2006). Photosynthetic biofilms naturally colonize the mine sediment. Metal exposure increased the exopolysaccharides (EPS) production in these biofilm (Priester *et al.*, 2006). The survival of biofilms under mixed-metal exposure has practical applications in the remediation of mine tailings (Garcia-Meza *et al.*, 2005).

2.3 Biofilm formation *in vivo*

Bacteria produce biofilm matrices based on various environmental conditions. Variation on temperature and nutrient content can greatly affect the biofilm formation *in vitro*. For example S. Typhimurium UMR1 is a temperature regulated strain which can produce cellulose and curli at 28ºC but not at 37ºC. With elevated levels of c-di-GMP, S. Typhimurium UMR1 can produce biofilm matrices at higher temperature (Kader *et al.*, 2006). The laboratory adapted strain in many cases can not produce biofilm at higher temperatures (Romling *et al.*, 2003). The bacterial capacity to form biofilm is studied mainly *in vitro* conditions. One important question is whether bacteria can produce biofilm *in vivo*.

It has been shown that *E. coli* used the bladder as a reservoir which was shown by microscopic examination. The quorum of bacterial cells is increased with the progression of time. It was shown that there is a shift of the cell shapes (Justice *et al.*, 2004; Justice *et al.*, 2006) and claimed that biofilm like pods are formed (Anderson *et al.*, 2003). It was also addressed that flagella aid in efficient urinary tract colonization (Wright *et al.*, 2005). Adhesion mediates the introduction of bacteria to the host and establish a life long relationship of uropathogenic *E coli* and the human urinary tract (Wright and Hultgren, 2006). Progression of bacterial colonization studied by real time (Mansson *et al.*, 2007) provides an account of the scenario *in vivo* which could be included to study the formation of Intracellular Bacterial Communities.

2.4 Cycles in biofilm formation

Biofilm is formed in several sequential stages after attachment to biotic or abiotic surfaces. After the initial settlement of the bacteria on the surfaces, bacteria can migrate over the surfaces. Within the biofilm, they form micro-colonies which are the basic structures. Bacteria express different kinds of biofilm matrices that firmly attach them to the surfaces. Over time the matured biofilm forms a 3D structure. S. Typhimurium produces cellulose and curli as the biofilm matrix components. C-di-GMP is regulating the transition from the planktonic state to

the biofilm state (Simm *et al.*, 2004). Once the biofilm is formed, it is not a static process but the switching between different phenotypes is a continuous process.

2.5 Biofilm matrix: description of different biofilm matrix:

Diverse groups of bacteria can produce different biofilm matrix components (Branda *et al.*, 2005; Romling *et al.*, 2003; Solano *et al.*, 2002; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). In S. Typhimurium, two well characterized biofilm matrices are cellulose (Solano *et al.*, 2002; Zogaj *et al.*, 2001) and curli (Barnhart and Chapman, 2006; Olsen *et al.*, 1989; Romling *et al.*, 1998a). Cellulose is known to be produced by plant. It has been shown that soil bacterium *Gluconoacetobaterium xylinus (G. xylinus)* can produce cellulose. Pathogenic bacteria like S. Typhimurium and *E. coli* have the cellulose operon and can synthesize cellulose (Solano *et al.*, 2002; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). The proteinaceous curli fibre is also an important biofilm matrix component which has impact on attachment to the epithelial cell (Wang *et al.*, 2006).

2.5.1 Cellulose

Cellulose is the most abundant biological polymer on earth and was first described by Anselme Payen in 1838. It is the major component of cell walls of higher plants and some algae (Kimura *et al.*, 2001). Cellulose production is a wide spread phenomenon in bacteria especially in *Enterobacteriaceae* including S. Typhimurium, *S. enteritidis*, *E coli*, *Citrobacter spp*., and *Enterobacter* spp (Romling *et al.*, 2003; Solano *et al.*, 2002; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). Protists, fungi, and tunicates also produce cellulose. *G. xylinus* is extensively studied as a model organism of cellulose biosynthesis. Studies on G. *xylinus* lead to the discovery of c-di-GMP as an allosteric activator for cellulose synthesis (Ross *et al.*, 1991). *G. xylinus* has been used as a model organism to study *in vitro* cellulose biosynthesis, purification of cellulose and identification of cellulose synthase (Kimura *et al.*, 2001; Ross *et al.*, 1991; Saxena *et al.*, 1994; Saxena and Brown, 2000).

Bacterial cellulose is used for multiple biomedical applications. Bacterial cellulose fibers produced by *G. xylinus* are used together with poly vinyl alcohol (PVA) to form biocompatible nanocomposite (Brown and Laborie, 2007). This nanocomposite can be made with similar mechanical properties of cardiovascular tissues such as aorta and heart valve leaflets (Millon and Wan, 2006). Novel and established implication of bacterial polysaccharides also revealed that bacterial cellulose is used in audio membranes (Sutherland, 1998).

2.5.2 Curli

Curli were first discovered in the late 1980s in *E. coli* strains that caused bovine mastitis (Olsen *et al.*, 1989). In *E.* coli and S. Typhimurium, they have been implicated in many physiological and pathogenic processes. Curli fibres are highly conserved in both organisms with respect to operon structure and regulation (Romling *et al.*, 1998a) and are involved in adhesion, aggregation and biofilm formation (Barnhart and Chapman, 2006). Host cell adhesion and invasion are also mediated by curli. Curli are the potent inducers of the host inflammatory response and release of cytokines (Wang *et al.*, 2006). Structurally and bio-chemically, curli belong to the class of fibre known as amoyloid and are responsible for a number of human diseases including Alzheimer's, Huntington's and prion diseases (Barnhart and Chapman, 2006). Many extracellular surface fibers produced by bacteria are important in

pathogenesis. There is evidence that curli are important in disease process. Curli binds to the extracellular matrix proteins fibronectin and laminin (Barnhart and Chapman, 2006). Curli interact with molecules of the immune system, major histocompatibility complex (MHC) class I which presents antigens to T cells, binds to curli (Olsen *et al.*, 1998).

2.5.3 Colanic acid

Colanic acid (CA) or M-antigen is an exo-polysaccharide produced by many enterobacteria including the majority of the *E. coli* strain (Meredith *et al.*, 2007). The colanic acid biosynthetic gene cluster in *E. coli* has been identified and found to be composed of 19 genes. Colanic acid forms a loosely associated saccharine mesh that coats the bacteria within the biofilm. Colanic acid comprises of repeating subunits and is believed to be expressed when bacteria is attached to an abiotic surface. Production of colanic acid is not necessary for initial attachment of the cells but for the subsequent three dimensional (3D) structure developments (Prigent-Combaret *et al.*, 2000) of bacteria, meaning that colanic acid plays a role after attachment of the bacteria. In S. Typhimurium, gene *wcaM* is involved in colanic acid biosynthesis and mutational inactivation of *wcaM* revealed that disrupted biofilm was formed but biofilm formation on a plastic surface was unaffected (Ledeboer and Jones, 2005) which indicates that biofilm formation by bacteria markedly varied on different kinds of surfaces.

2.5.4 Antigen 43

A group of proteins that are represented by the auto-transporter subgroup of proteins are antigen 43 (Ag43). Ag43 is a self recognizing adhesin associated with cell aggregation and biofilm formation in *E. coli* (Schembri *et al.*, 2003). The sequenced genome of the prototype UPEC strain CFT073 contains two variant of Ag43 in encoding genes in the pathogenicity island. Ag43a produced strong aggregation phenotype and promoted biofilm formation (Ulett *et al.*, 2007). Sequence analysis did not show any homologus gene encoding Antigen 43 in S. Typhimurium.

3.0 rdar morphotype system for studying multicellular behaviour

3.1 rdar morphotype and biofilm matrix

Bacteria expressing cellulose and curli show a red, dry and rough (rdar) phenotype when grown on agar plates containing the diazo dye Congo Red (CR). It provides a simple and convenient way to identify genes important for cellulose (Solano *et al.*, 2002; Zogaj *et al.*, 2001) and curli (Collinson *et al.*, 1996; Romling *et al.*, 1998a) expression. S. Typhimurium, *E. coli* and many other members of *Enterobacteriaceae* express the polysaccharide cellulose as a second extracellular matrix components (Bokranz *et al.*, 2005; Solano *et al.*, 2002; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). The spectrophotometric property of Congo red dye is changed due to the binding of cellulose and curli to CR. Bacteria that only express cellulose show a characteristic pink, dry and rough phenotype (pdar). Bacteria expressing curli show a characteristic brown, dry and rough (bdar) phenotype on Congo Red plate (Romling *et al.*, 1998a; Romling *et al.*, 1998b; Romling *et al.*, 2000). Bacteria that express none of the components have a smooth and white (saw) phenotype. The other components of the biofilm matrix for example, the extracellular polysaccharides (EPS), capsule, lipopolysaccharides (White *et al.*, 2003) and large secreted protein bapA (Latasa *et al.*, 2005) do not show a distinct phenotype in CR plates. Agar medium containing the dye was previously used in different studies to differentiate between virulent and avirulent colonies of *Shigella*, *V. cholerae*, *E. coli* and *Neiserria meningitides* (Payne and Finkelstein, 1977; Qadri *et al.*, 1988).

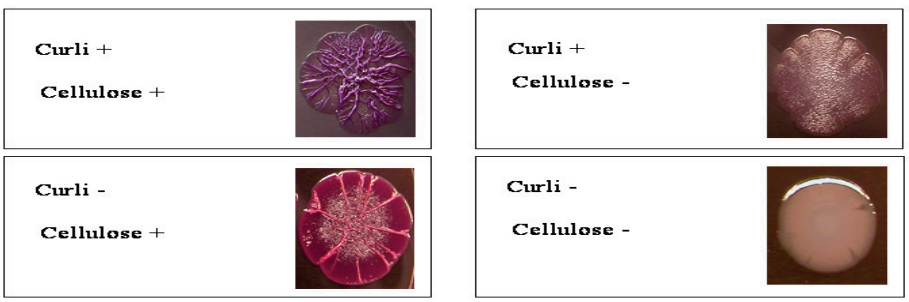

Figure 1. S. Typhimurium, UMR1 showing different morphotype at low temperature (28ºC), (picture courtesy Dr. Xhavit Zogaj, PhD thesis 2004). Upper panel left showed rdar phenotype which expressed cellulose and curli and right showed a bdar phenotype which expressed only curli. Lower panel left showed a pdar phenotype which expressed cellulose only while right showed a saw phenotype which expressed none of the biofilm matrix components (Romling *et al.*, 1998a; Romling *et al.*, 1998b; Romling *et al.*, 2000; Zogaj *et al.*, 2001).

3.2 CsgD- a central regulator for biofilm formation

CsgD is the master regulator for biofilm matrix component in S. Typhimurium and *E. coli* (Gerstel *et al.*, 2003; Gerstel and Romling, 2003; Gerstel *et al.*, 2006). Chromosomal inactivation of CsgD leads to a saw phenotype where none of the biofilm matrices are expressed (Romling *et al.*, 2000). CsgD is a transcriptional activator for AdrA. In the temperature regulated strain of S. Typhimurium UMR1, CsgD is not expressed at higher temperature (37ºC). The temperature regulation of CsgD can be overcome by over-expressing GGDEF domain proteins (Kader *et al.*, 2006). A single point mutation in CsgD promoter can also over-come the temperature regulation of CsgD (Romling *et al.*, 1998b). CsgD is categorized in the LuxR super family of transcriptional regulators carrying an N-terminal receiver domain and a C-terminal DNA binding helix –turn-helix domain. The expression of CsgD is complex and regulated by many environmental conditions like temperature, osmotic pressure, nutrient starvation and oxygen tension (Gerstel and Romling, 2001; Gerstel *et al.*, 2003). In *Salmonella* capsular polysaccharide and the large secreted protein BapA are other surface associated structures that are positively regulated by CsgD (Gibson *et al.*, 2006; Latasa *et al.*, 2005). It was shown that expression of cellulose is a common feature of *E. coli* and almost 50% of tested isolates express cellulose in a screen. Interestingly, it was shown that the regulatory cascade of cellulose in commensal *E. coli* is different than in S. Typhimurium. The regulator CsgD is not required for cellulose production and it does not require GGDEF domain protein AdrA as well (Da Re and Ghigo, 2006). It has shown that cellulose expression was independent of CsgD and AdrA expression in *E. coli*. The study also suggests that a GGDEF domain protein YedQ is responsible for activating the expression of cellulose (Da Re and Ghigo, 2006).

3.3 AdrA and YhjH– candidate GGDEF and EAL domain proteins

There are 12 GGDEF and 14 EAL domain proteins in S. Typhimurium. It has shown that a subset of these domain proteins are involved in rdar morphotype development, motility phenotype expression, biofilm formation, CsgD and CsgA regulation (Garcia *et al.*, 2004; Kader *et al.*, 2006; Romling, 2005; Simm *et al.*, 2004; Simm *et al.*, 2007; Weber *et al.*, 2006). AdrA (STM0385) was studied as a GGDEF domain protein in S. Typhimurium. AdrA contributes to the production of c-di-GMP, which interacts with bacterial cellulose synthase A (*bcsA*), a direct target for c-di-GMP (Ryjenkov *et al.*, 2006) and induces the expression of cellulose. C-di-GMP up-regulates the central regulator CsgD through over-expression of GGDEF domain protein (Kader *et al.*, 2006). It was shown that by over-expressing AdrA, the biofilm phenotype can be restored in HmsT (a GGDEF domain protein) mutant of *Y. pestis* (Simm *et al.*, 2005). The HmsT, a GGDEF domain protein from *Y. pestis* can restore cellulose in AdrA knock-out mutant of S. Typhimurium. AdrA contributes 60% of the basal level of c-di-GMP in S. Typhimurium whereas there is no effect on CsgD when AdrA is chromosomally inactivated. It means that there might exist micro-compartments for c-di-GMP inside the cell (Kader *et al.*, 2006).

Among the 14 EAL domain proteins, YhjH (STM3611) is studied as an EAL domain protein. YhjH is known from the study of Ko and Park to regulate flagella mediated motility in *E. coli* (Ko and Park, 2000). YhjH is responsible for c-di-GMP degradation in S. Typhimurium. It has been shown that YhjH has an opposing effect of the GGDEF domain protein in bacterial transition from sessility to motility (Simm *et al.*, 2004; Simm *et al.*, 2007).

3.4 The regulatory network of rdar morphotype expression

Analysis of the molecular regulation of biofilm matrices have been depicted from many studies (Gerstel *et al.*, 2003; Kader *et al.*, 2006; Romling *et al.*, 1998b; Romling *et al.*, 2000). CsgD is the master regulator for both the biofilm matrix components and GGDEF domain proteins up-regulate cellulose and curli by over-producing c-di-GMP (Kader *et al.*, 2006; Simm *et al.*, 2004; Weber *et al.*, 2006).

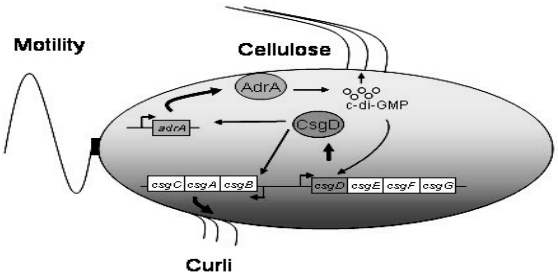

Figure 2. Regulation of biofilm formation at lower temperature (28ºC) in S. Typhimurium UMR1. The model illustrates the rdar morphotpe development where CsgD directly activates the transcription of *csgBAC* operon (Romling *et al.*, 2000). It results in expression of *csgA* and *csgB*. CsgD also activates the transcription of *adrA*, which is a diguanyl cyclase and synthesizes c-di-GMP (Romling *et al.*, 2000). C-di-GMP is up-regulating cellulose and curli (Kader *et al.*, 2006; Simm *et al.*, 2004). CsgD is up-regulated with higher production of c-di-GMP (Kader *et al.*, 2006).

4.0 GGDEF-EAL domain proteins

4.1 GGDEF and EAL domain proteins in diverse groups of bacteria

Mass genome sequencing revealed that a lot of proteins share different domains known as domain of unknown functions 1 (DUF1) and domain of unknown function 2 (DUF2) (Galperin *et al.*, 2001). The DUF1 domain is known as GGDEF domain and DUF 2 domain as EAL domain. Generally a GGDEF domain is approximately 180 amino acids and an EAL domain is 250 amino acids long (Galperin *et al.*, 2001). The GGDEF domain was first described in *Caulobacter crescentus* by Hecht and Newton. It has been shown that the GGDEF domain protein PleD was involved in transition from swarmer cells to stalk forming cells which was the first indication that GGDEF domain protein is involved in bacterial signal transduction (Hecht and Newton, 1995). Most free living bacteria harbor GGDEF and EAL domain containing proteins (Galperin *et al.*, 2001; Rashid *et al.*, 2003). It was assumed that free living bacteria might use these proteins to counter the adverse environmental circumstances. The proteins are assigned for specific task distribution in multicellular development (Garcia *et al.*, 2004; Kader *et al.*, 2006). The significant impact of GGDEF domain protein AdrA was investigated when it was shown that this protein is involved in cellulose biosynthesis in S. Typhimurium (Romling *et al.*, 2000; Zogaj *et al.*, 2001). The mechanisms of cellulose production and molecular regulation were not evident. It was known that the second messenger molecule c-di-GMP up-regulates the cellulose biosynthesis through the diguanyl cyclase activity. The expression of cellulose was down regulated through phosphodiesterase activity that degrades c-di-GMP (Amor *et al.*, 1991; Tal *et al.*, 1998). Through a complementation study, it was shown that the GGDEF domain protein can induce cellulose. The genetic data indicates that GGDEF domain proteins are responsible for c-di-GMP synthesis (Ausmees *et al.*, 2001). The direct evidence of involvement of c-di-GMP in cellulose synthesis was known (Amor *et al.*, 1991; Ross *et al.*, 1991; Tal *et al.*, 1998). The subsequent study from the Urs Jenal laboratory showed that the GGDEF domain containing protein PleD can synthesize c-di-GMP (Chan *et al.*, 2004; Jenal, 2004; Paul *et al.*, 2004). At the same time, studies on S. Typhumirum showed that GGDEF and EAL domain are involved in c-di-GMP turn over (Simm *et al.*, 2004).

GGDEF and EAL domain proteins were studied extensively in different laboratories. The functions of these proteins are known from different studies showing that c-di-GMP is implicated in multiple targets (Garcia *et al.*, 2004; Kader *et al.*, 2006; Romling *et al.*, 2005; Romling and Amikam, 2006) and has a wide range of biological functions (Kader *et al.*, 2006; Simm *et al.*, 2004; Simm *et al.*, 2007).

4.2 Distribution of GGDEF-EAL domain proteins and putative roles

Most bacteria harbor more than one GGDEF or EAL domain protein, the highest number of GGDEF and EAL domain proteins is in *V. vulnificus* (66 GGDEF and 33 EAL) (Romling *et al.*, 2005). Large numbers of GGDEF and EAL domain proteins were initially believed to be redundant in the chromosome. Systematic studies revealed that these large numbers of proteins have specific task distribution in bacteria. In S. Typhimurium, the phenotypic and functional inventory was made for all the GGDEF and EAL domain proteins in development of multicellular behavior. It was shown that a subset of the proteins are involved in rdar morphotype development , regulation and other phenotype expression (Kader *et al.*, 2006; Simm *et al.*, 2007). The functions of the GGDEF and EAL domain was investigated by system biology approach in *Pseudomonas aeruginosa*. A comprehensive survey was

conducted for the roles of GGDEF and EAL domain proteins in two commonly studied *P. aeruginosa* strains. A subset of the GGDEF and EAL domain proteins showed the diguanyl cyclase and phosphodiesterase activity even though there were a vast majority were not assigned any of these activities (Kulasakara *et al.*, 2006). Interestingly the cytotoxicity and biofilm formation phenotype was mediated by a subset of the GGDEF and EAL domain proteins but there was no inverse correlation.

4.3 Cyclic Diguanosine Monophosphate

The di nucleotide c-di-GMP has been known more than two decades. The effect of this molecule in cellulose biosynthesis was described in pioneering works from Benziman laboratory (Amor *et al.*, 1991; Tal *et al.*, 1998; Weinhouse *et al.*, 1997). Genetic data has indicated that proteins containing GGDEF domain posses diguanyl cyclase activity (Ausmees *et al.*, 2001). There are multiple GGDEF domain proteins encoded in the chromosome of many bacteria which contribute to the basal level of c-di-GMP. It is generally accepted that GGDEF domain proteins are diguanyl cyclase, EAL and HD-GYP domain proteins are phosphodiesterases (Kulasakara *et al.*, 2006; Romling, 2005; Ryan *et al.*, 2006).

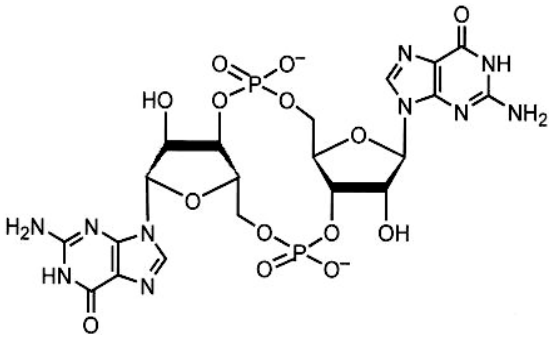

Figure 3. Structure of cyclic di nucleotide, c-di-GMP

4.4 Diguanyl cyclase and phosphodiesterases

Synthesis of c-di-GMP from two molecule of GTP occur in two steps with ppGpG as an intermediate and each step releasing pyrophosphate that ultimately hydrolyzed to inorganic phosphate (Chan *et al.*, 2004). The degradation of c-di-GMP also occurs in two steps where pGpG is an intermediate. GGDEF domain proteins are playing a role in the synthesis of c-di-GMP as diguanyl cylcase and EAL and HD-GYP domain degrade it through phosphodiesterase activity.

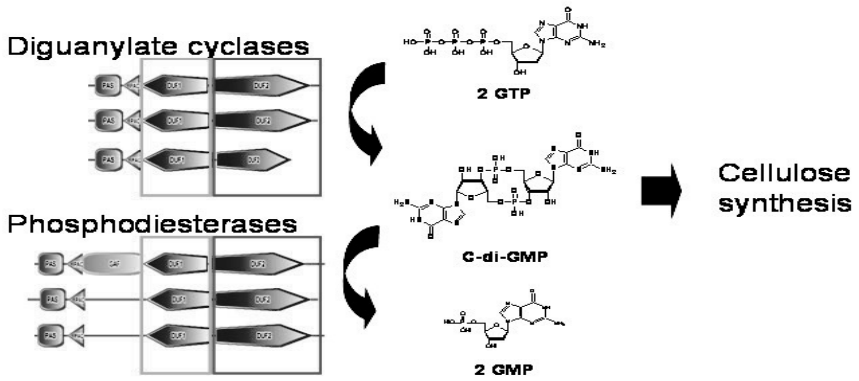

Figure 4. Mechanism of action of synthesis and degradation of c-di-GMP by GGDEF and EAL domain proteins in *G. xylinus*.

4.5 C-di-GMP targets

Among the genes in *bcsABZC* operon, *bcsA* encodes the cellulose synthase which is the active subunit of the cellulose synthesizing complex (Zogaj *et al.*, 2001). C-di-GMP is regulating the expression of cellulose by binding to the cellulose synthase BcsA (Ryjenkov *et al.*, 2006). The list of new targets for c-di-GMP is increasing. Over production of c-di-GMP in S. Typhimurium showed up-regulation of whole rdar morphotype. Molecular analysis of rdar morphotype up-regulation indicates that the structural gene for curli biosynthesis, *csgA* is up-regulated. The up-regulation of CsgA is controlled by c-di-GMP via modulation of CsgD expression (Kader *et al.*, 2006). C-di-GMP is regulating the virulence gene expression (Tischler and Camilli, 2005) and it negatively regulates cholera toxin production (Tamayo *et al.*, 2005; Tischler *et al.*, 2002; Tischler and Camilli, 2004).

4.6 Role of exogenous c-di-GMP in eukaryotic cells and biofilm formation

C-di-GMP has been shown to interact with eukaryotic signalling system including the inhibition of growth proliferation of different cancer cell lines *in vitro (Amikam et al., 1995; Steinberger et al., 1999)***.** When the lymphoblastoid CD4+ Jurkat cell line was exposed to c-di-GMP, the cells exhibited a marked elevated level of CD4 receptor up to 6.3 fold. C-di-GMP also caused a marked blockage of the cell proliferation at S- phase (Steinberger *et al.*, 1999). Growth of the colon cancer cell line H508 was also inhibited by c-di-GMP without cytotoxicity (Karaolis *et al.*, 2005a). Treatment of c-di-GMP from external sources also found to be effective against cell to cell communication, biofilm formation as well as virulence in *Staphylococcus aureus* (Brouillette *et al.*, 2005; Karaolis *et al.*, 2005b). Further investigation showed that c-di-GMP acts as an immunostimulatory molecule which opens possibilities for

this molecule in multiple applications (Karaolis *et al.*, 2007). It was shown that c-di-GMP could be used as a potent mucosal adjuvant (Ebensen *et al.*, 2007a; Ebensen *et al.*, 2007b)

4.7 Regulation of virulence gene expression by c-di-GMP

C-di-GMP is playing a major role in virulence attributes of different bacteria including *V. cholerae* (Tischler and Camilli, 2005), *X. campestris* (Ryan *et al.*, 2007) and *P. aeruginosa* (Kulasakara *et al.*, 2006). The VieSAB three-component system in *V. cholerae* is required for maximal expression of *ctxAB* which encodes the cholera toxin. VieA is a c-di-GMP specific phosphodiesterase which maintain a low c-di-GMP level (Tamayo *et al.*, 2005). The decrease of c-di-GMP by VieA positively regulates the transcription of the transcriptional regulator ToxT that directly activates the expression of *ctxAB* (Tischler *et al.*, 2002). It has been shown that biofilm formation and virulence gene expression are inversely regulated in *V. cholerae*. The chromosomal inactivation of VieA increased the biofilm formation through up-regulated transcription of Vibrio exo-polysaccharide synthesis (VPS) gene by the transcriptional activator VpsR (Tischler and Camilli, 2004). It is suggested that the persistence in the environment and survival within the host can be also modulated inversely by c-di-GMP concentration (Tamayo *et al.*, 2007). In *Bordetella pertussis*, the EAL domain proteins, BvgR is required for virulence in the mouse aerosol model (Merkel *et al.*, 1998).

In S. Typhimurium, *in vivo* screen for the genes responsible for withstanding oxidative stress by phagocytes has discovered STM1344, an unconventional EAL domain protein. The mutant of STM1344 can kill the macrophages at an earlier time point and are more cytotoxic than the wild type (Hisert *et al.*, 2005).

5.0 Polynucleotide phosphorylase and multicellular behaviour

5.1 Roles of Polynucleotide phosphorylase

Polynucleotide phosphorylase (PNPase) was discovered while exploring the possible mechanism of aerobic phosphorylation in bacterial extract (Kornberg, 2001). The initial impression was that PNPase is responsible for biosynthesis of ribonucleic acid (RNA), in that PNPase was able to catalyze polymerization of ribonucleotides (Kornberg, 2001). At physiological concentration of phosphate, however PNPase acts as a phosphorolytic exoribonuclease that plays a central role in RNA processing in bacteria and plants (Sarkar and Fisher, 2006). The human polynucleotide phosphorylase (hPNPase) structurally and biochemically resembles PNPase of other. PNPase is the part of the RNA degradosome which is a bacterial protein machine devoted to RNA degradation and processing. In *E. coli*, it is typically composed of the endoribonuclease RNAse E which serves as a scaffold for the other components, the RNA helicase RhlB, enolase and exoribonuclease PNPase (Lin and Lin-Chao, 2005). There are several other proteins that are associated with the core complex. It is not known whether in most cases such proteins are occasional contaminants or specific components. The functions of those proteins are also not known.

5.2 Domain architecture and functions of PNPase

PNPase structure analysis has been performed in *Streptomyces antibioticus* which reveals the presence of five domains. They include two ribonuclease PH (RPH)- like pnp1 and pnp2, one alpha helical, one KH and one S1 domain. The trimeric nature of PNPase was also confirmed. pnp 1 and pnp 2 are closely related functionally and in sequence similar to the RPH domain. Phylogenetic analysis of the gene geneology of pnp1, pnp2 and RNase PH (RPH) domain suggests that PNPase was formed via a duplication process that also produced the RPH protein. This duplication most likely occurred in the common ancestor of the three oganismal super kingdoms, Archaea, Eukarya and bacteria (Leszczyniecka *et al.*, 2004). Pnp2 and RPH domain are more closely related to each other than either the two to pnp1, suggesting a deeper differentiation of pnp 1 in the common ancestor. Functional studies revealed that KH and S1 RNA binding domain are involved in pnp mRNA recognition (Leszczyniecka *et al.*, 2004). The S1 and KH domain can be released from the enzyme by mild proteolysis or by truncation of the gene. It was shown that deletion of the S1 domain reduces the apparent activity of the enzyme by almost 70 fold under low ionic strength conditions (Stickney *et al.*, 2005). It also limits the enzyme to digest a single substrate molecule. The activity and product release was highly regained at higher ionic strength. The deletion of the S1 domain also reduces the affinity of the enzyme for RNA without affecting the enzymes ability to bind RNase E. Deletion of the KH domain also showed a similar but less severe effect. Knocking out of both KH and S1 domain showed loss of activity and product release, RNA binding had been attenuated. The loss of KH and S1 domain did not show any effect on binding to RNase E (Stickney *et al.*, 2005). Further analysis revealed that the effect of PNPase on T3SS is independent of its ribonuclease activity and instead requires its S1 RNA binding domain. The wild type like T3SS function was possible to reconstitute in the PNP mutant by expressing approximately 70 amino acids of the S1 domain from either PNPase, RNase R, RNaseII or RpsA (Rosenzweig *et al.*, 2005).

5.3 PNPase and bacterial adaptation in different environment

In order to survive in adverse environmental conditions, bacteria maintain different adaptation mechanism to encounter the stress. In many species of bacteria, stresses like low temperature and encounters with phagocytes have been well studied. It was shown that PNPase is required for growth of *Y. pestis* at low temperature (Rosenzweig *et al.*, 2005). Biofilm has gained an increased appreciation as a primordial prokaryotic response to environmental cues. It was shown that *Y. pestis* is capable of forming biofilm at lower temperature by expressing GGDEF domain protein HmsT (Simm *et al.*, 2005). The mutational inactivation of EAL domain proteins HmsP in *Y. pestis* gives rise to biofilm phenotype at lower temperature (Kirillina *et al.*, 2004). In *Y. pestis* and *Yersinia. pseudotuberculosis*, it was shown that PNPase enhances the ability to counter the killing activities of murine macrophages (Rosenzweig *et al.*, 2005). PNPase is also required for the optimal activity of type three secretion systems (T3SS). This organelle helps to inject the effectors proteins directly into host cells. The PNPase mutant was also found to be less virulent in *Y. pestis* (Rosenzweig *et al.*, 2007). It is suggested that PNPase is playing pleotrophic roles in enhancing *Y. pestis* to survive in stressful conditions.

The growth of *E. coli* is transiently halted when cold shock is encountered and the specific cold shock proteins (CSPs) are greatly induced (Beran and Simons, 2001; Yamanaka and Inouye, 2001). Due to the presence of high number of CspA family *E. coli* is highly protected from cold shock (Xia *et al.*, 2001). It was shown that PNPase is required for the down regulation of CSP in *E. coli*. PNPase mutant plays a significant role in maintaining the level of CSP when cold shock is applied. It is evident that PNPase and poly A polymerase are important for cold shock adaptation (Yamanaka and Inouye, 2001). Psychotrophic bacterium *Yersinia enterocolitica* also holds the temperature dependent adaptation. Investigation on the underlying mechanism for cold adaptation revealed that PNPase is involved in cold temperature adaptation (Goverde *et al.*, 1998). The PNPase encoding gene mutant in *Pseudomonas putida* did not show any cold sensitivity (Favaro and Deho, 2003).

It was shown that in S. Typhimurium that mutational inactivation of this cold shock associated exoribonuclease PNPase enable the bacteria to cause chronic infection at 37ºC. It has also affected the bacterial replication in mice (Clements *et al.*, 2002). PNPase inactivation also results in increased expression of *Salmonella* plasmid virulence (*spv*) gene expression (Ygberg *et al.*, 2006). A single point mutation in PNPase effects complete inactivation of PNPase activity which affected bacterial invasion and intracellular replication. This effect determines the alternation between acute or persistence infection in a mouse model for S. Typhimurium (Clements *et al.*, 2002).

5.4 Role of PNPase in Salmonella Pathogenecity Island (SPI) and Salmonella Plasmid virulence (SPV)

Through microarray analysis, PNPase was found to affect the mRNA levels of a subset of virulence genes, especially the Salmonella Pathogenecity Island I (SPI I) and (SPI II) (Clements *et al.*, 2002). These results suggest a connection between PNPase and Salmonella virulence. It was assumed that PNPase activity can represent a strategy for the establishment of persistency. The whole genome microarray analysis also revealed that expression of Salmonella Plasmid Virulence (*spv*) genes expression was also induced in PNPase mutant. The investigation also showed that including *spvABC*, six genes are significantly up-regulated. Mutational inactivation of the regulator for *spv* genes, *spvR* diminishes the increased expression of *spv* (Ygberg *et al.*, 2006).

6.0 Aims of the thesis

The objective of this thesis was to delineate the role of GGDEF and EAL domain proteins in bacterial physiology.

Specific aims were

I. Investigation of novel biological functions of the second messenger molecule c-di-GMP.
II. Elucidation of the task distribution of different GGDEF-EAL domain proteins and molecular regulation of c-di-GMP
III. Analysis of the inter-connection between c-di-GMP signalling and regulation of PNPase.

7.0 Results and discussion

7.1 Paper I:

GGDEF and EAL domains inversely regulate cyclic di GMP levels and transition from sessility to motility.

Although many bacterial proteins share the conserved regions known as GGDEF and EAL domains (Galperin *et al.*, 2001), the functions of these domains were unknown. GGDEF domain was thought to be involved in production of c-di-GMP and EAL domain in the degradation of c-di-GMP. Earlier studies showed that bacteria like S. Typhimurium and *E. coli* can produce cellulose as a biofilm matrix component (Solano *et al.*, 2002; Zogaj *et al.*, 2001). Among the 12 GGDEF domains containing proteins in the chromosome of S. Typhimurium AdrA, was identified as the regulator of cellulose expression. AdrA has an N-terminal MASE2 domain and a C-terminal GGDEF domain. YhjH does not have any other domain except EAL domain. It was shown in *G. xylinus* that the concentration of the allosteric activator of cellulose synthase, c-di-GMP can be controlled with diguanyl cylcase or phosphodiesterase activity (Tal *et al.*, 1998). The abundance of GGDEF and EAL domain proteins in different organisms has brought the prediction that c-di-GMP could be a generalized messenger molecule.

In this study, there were two broad questions that were asked. The first question was- whether GGDEF and EAL domain containing proteins are involved in synthesis and degradation of c-di-GMP. The second question dealt with the biological relevance and novel functions of c-di-GMP. To answer these questions, two candidate proteins were selected from set of GGDEF and EAL domain proteins. STM0385 which is known as AdrA, was described earlier to show an effect in cellulose production (Zogaj *et al.*, 2001). STM3611 which is known as YhjH, was previously shown to regulate the motility phenotype in *E. coli* (Ko and Park, 2000). These genes were cloned in a vector under control of the tightly regulated arabinose inducible promoter so that expression of these genes could be regulated by modulating the arabinose concentration.

To investigate whether GGDEF domain can induce cellulose production, AdrA was over expressed in S. Typhimurium. An *adrA* and *csgBA* double mutant background was used. It was shown that AdrA can induce cellulose and the phenotype correlates with the level of induction. Over-expression of AdrA in a cellulose synthase mutant showed that c-di-GMP can not induce cellulose. The same panels of experiments were performed with YhjH. Over-expression of YhjH down regulated the whole rdar morphotype to almost saw morphotype. To find out whether AdrA is involved in c-di-GMP synthesis and YhjH is degrading it, a high pressure liquid chromatography (HPLC)-mass spectrometry (MS) based method was developed. With the help of this technique, it was shown that over-expression of AdrA leads to a substantial increase in the amount of c-di-GMP within the cell. On the other hand, strains co-expressing AdrA and YhjH have reduced amounts of c-di-GMP. To confirm that GGDEF motif is responsible for the synthesis of c-di-GMP and EAL motif is responsible for degradation of c-di-GMP, we performed a mutational analysis for the GGDEF and EAL motif. Two point mutations were introduced to the GGDEF motif. The GG was changed to AA resulting AADEF motif. Over expression of mutated GGDEF domain protein AdrA can not induce cellulose. Analysis of c-di-GMP showed that mutated AdrA can not produce c-di-GMP. In the case of YhjH, a glutamate was mutated to an alanine that changed to an inactive

form of the protein that could not down regulate the c-di-GMP. These experiments have shown that GGDEF domain has diguanyl cyclase activity and EAL domain has phosphodiesterase activity. The findings are in agreement with the earlier prediction where GGDEF domain was suggested to synthesise c-di-GMP (Ausmees *et al.*, 2001). It was shown that the GGDEF domain of PleD protein in *C. crescentus* is a diguanyl cyclase (Paul *et al.*, 2004). The biochemical and structural analysis of the GGDEF domain showed that GGDEF motif is part of the active site of GGDEF domain. Mutation in any of the amino acids to alanine in the motif resulted in an enzymatically inactive protein (Chan *et al.*, 2004; Ryjenkov *et al.*, 2005). The proposed mechanism of action for converting GTP to c-di-GMP includes phosphorylation induced dimerization of two substrate binding PleD monomers, bringing the GGDEF and GTP complex into close contact, which ultimately catalyzes the condensation of the GTP molecules into c-di-GMP (Chan *et al.*, 2004; Paul *et al.*, 2007).

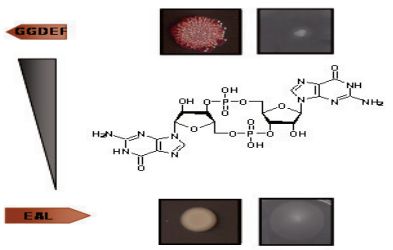

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Figure 5. Over-expression of GGDEF domain proteins lead to the higher production of second messenger molecule c-di-GMP. C-di-GMP up-regulates the rdar phenotype and down-regulates motility phenotype in S. Typhimurium (Simm *et al.*, 2004). Conversely, over-expression of EAL domain proteins down regulated c-di-GMP and rdar phenotype while up-regulated motility phenotype (Rahman *et al.*, 2007; Romling, 2005; Simm *et al.*, 2004; Simm *et al.*, 2005).

Although the functions of EAL domain containing proteins have been shown in different bacteria, the active site of the EAL domain protein has not been identified. The biochemical analysis of several EAL domain containing proteins have been performed which indicates that proteins containing EAL domain have c-di-GMP specific phosphodiesterase activity (Christen *et al.*, 2005; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005). It was shown that c-di-GMP hydrolysis is catalysed by monomeric EAL domain protein and the major product is a linear di-GMP molecule (l-di-GMP) (Schmidt *et al.*, 2005). Further hydrolysis of the second phosphodiester bond of l-di-GMP to GMP appears to be unspecific.

Most of the biochemical mechanisms of the compound c-di-GMP were shown in the context of polysaccharide biosynthesis. GGDEF, EAL and HD-GYP domain were shown to be involved in modulating c-di-GMP concentration. In this study, we have shown the biological functions of the c-di-GMP since c-di-GMP is regulating the cellulose expression; we have further proved that it has significant impact on phenotype like bacterial biofilm formation in S. Typhimurium. The effect is even seen when the biofilm formation is investigated in minimal medium. We have shown that the effect of GGDEF and EAL domain proteins on biofilm formation also inversely regulated in S. Typhimurium. This trend is also followed

when different medium was used. The effect was investigated in *E. coli* and *P. aeruginosa* to show that it is a generalized event in bacteria. The effect on biofilm formation by these GGDEF and EAL domain proteins were shown in diverse groups of bacteria including *Aeromonas veronii* biovar sobria, *Shewanella oneidensis* and *P. aeruginosa* (Kazmierczak *et al.*, 2006; Rahman *et al.*, 2007; Thormann *et al.*, 2006).

Bacteria show motility phenotype mediated by flagella and type IV pili. S. Typhimurium and *E. coli* show the flagella mediated motility known as swimming and swarming. *P. aeruginosa* show type IV pili mediated twitching. We have shown that swimming and swarming are two phenotypes which are regulated by c-di-GMP. When level of c-di-GMP is increased with over-expression of the GGDEF domain protein AdrA, bacteria can hardly move, but bacteria are more motile when there is a low level of c-di-GMP through over-expression of the EAL domain protein YhjH. To show that c-di-GMP has a global impact in motility phenotype, we have over-expressed the AdrA and YhjH in *E. coli* and *P. aeruginosa*. We have shown a similar effect of c-di-GMP on motility phenotype in both the organisms. Studies on different organisms showed the same effects in agreement with our findings (Kazmierczak *et al.*, 2006; Kuchma *et al.*, 2007; Rahman *et al.*, 2007; Thormann *et al.*, 2006).

C-di-GMP is established now as a secondary messenger molecule which regulates multiple phenotypes and cellulose biosynthesis (Garcia *et al.*, 2004; Kader *et al.*, 2006; Rahman *et al.*, 2007; Rashid *et al.*, 2003; Simm *et al.*, 2004; Tischler and Camilli, 2004). The effect of c-di-GMP is now widely accepted in diverse group of bacteria which is predicted to be useful for different applications (Brouillette *et al.*, 2005; Ebensen *et al.*, 2007a; Karaolis *et al.*, 2005a; Karaolis *et al.*, 2005b; Karaolis *et al.*, 2007). The novel phenotypes regulated by GGDEF and EAL domain protein is an important direction for the current investigation. It is also interesting to investigate the effect of GGDEF and EAL domain proteins in the environment. It is not clear why the bacteria are harboring both EAL and HD-GYP domain which have the same apparent functions.

7.2 Paper II

Phenotypic convergence mediated by GGDEF-domain- containing proteins

Yersinia pestis is the causative agent of plague. It has evolved an arthropod-borne route of transmission, infecting fleas and mammalian hosts. Both virulence and transmission factors are crucial for the development of the disease. Transmission of plague by fleas depends on infection of the proventicular valve in insect's foregut by a dense aggregate of *Y. pestis*. For this infection hemin storage (*hms*) genes are required. It was shown that *hms* genes are required for production of extracellular matrix as well as biofilm development (Jarrett *et al.*, 2004). Hms (+) phenotype of *Y. pestis* promotes the binding of haemin or Congo Red to the cell surface. The regulation of the phenotype does not occur at the transcriptional level, instead, proteins of *hms* operon are degraded at a higher temperature (Kirillina *et al.*, 2004). It is predicted that biofilm development is a prerequisite for the transmission of plague. Biofilm formation represent a possible mechanism of transmission because biofilms are more resistant to human polymorphonuclear leukocyte clearance (Jarrett *et al.*, 2004).

The molecular mechanism of the CR binding phenotype was established in *Y. pestis*. The phenotype related to EAL domain proteins were shown in EAL domain protein HmsP. HmsP encodes a putative phosphodiesterase and mutational inactivation gives rise to red colonies on

CR plates indicating biofilm formation. A GGDEF domain protein HmsT also showed extensive biofilm formation when it is over expressed (Kirillina *et al.*, 2004).

AdrA, the GGDEF domain protein from S. Typhimurium is known to be involved in cellulose biosynthesis (Simm *et al.*, 2004; Zogaj *et al.*, 2001). Inactivation of AdrA leads to a change of wild type rdar morphotype to bdar morphotype on CR plates. We also know that AdrA can produce c-di-GMP and regulate multiple phenotypes (Simm *et al.*, 2004). In this study we have shown that GGDEF domain from diverse bacteria like *Y. pestis* can cross complement the GGDEF domain in S. Typhimurium and reconstitute the function of the GGDEF domain protein.

The GGDEF domain protein HmsT in *Y. pestis* was previously characterized (Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 2004). It was known that HmsT is related to the biofilm phenotype. We have over-expressed HmsT in an *adrA* and *csgA* knockout double mutant in S. Typhimurium. It was shown that up-regulation of HmsT can up-regulate the cellulose in S. Typhimurium. The result indicates that at lower temperature cellulose can be up-regulated by HmsT protein in S. Typhimurium. The CR plate phenotype was further confirmed by calcoflour binding assay. This finding is in agreement with the previous finding that HmsT is involved in biofilm formation at lower temperatures in *Y. pestis* (Kirillina *et al.*, 2004). The HmsT protein was also over-expressed in a *bcsA* knock-out background to show that the cellulose synthase BcsA is crucial for the phenotypic complementation in S. Typhimurium (Simm *et al.*, 2005).

The potential of the GGDEF domain protein AdrA to induce the biofilm in *Y. pestis* was investigated in an *hmsT* mutant. The *hmsT* mutant does not show significant biofilm formation in liquid culture. The biofilm forming capacity was monitored on borosilicate glass test tubes at lower temperatures. The result indicates that when AdrA is over-expressed and was not induced by arabinose, the bacteria can not produce biofilm. On the other hand when AdrA was induced by arabinose, the bacteria produce higher amount of biofilm. The biofilm was stained with crystal violet which was quantified after dissolving the biofilm with dimethyl sulfoxide (DMSO). Confocal laser scanning images of green fluorescent protein expressing *Y. pestis* harboring HmsT and AdrA showed that these proteins are crucial for biofilm formation. AdrA can cross complement the functions of HmsT in *Y. pestis*.

The biofilm forming capacity of S. Typhimurium expressing HmsT was shown on CR plates. It was shown that the pdar phenotype is up-regulated. Further analysis of the phenotype on calcoflour plate showed that cellulose is up-regulated in S. Typhimurium harboring *hmsT*. In liquid culture at lower temperature, it also showed the potential to express biofilm. It was also investigated whether HmsT can up-regulate the phenotype in different media. It was shown that HmsT has the capacity to express biofilm in M9 minimal medium. The quantification of biofilm after dissolving with DMSO showed that the biofilm is higher than the vector control background.

GGDEF domain proteins synthesize the secondary messenger molecule c-di-GMP which up-regulates biofilm. It was predicted that HmsT might have diguanyl cyclase activity. The expression of the protein showed positive regulation of biofilm phenotypes. To investigate that HmsT can produce c-di-GMP when over-expressed in S. Typhimurium, we have analyzed the nucleotide extracts. It was found that there is an increased level of c-di-GMP in S. Typhimurium harboring HmsT.

It is not known how bacteria acquire such larger numbers of GGDEF and EAL domain proteins from evolutionary perspectives. Further studies showed that ubiquitous gram-negative *Aeromonas veronii* biovar sobria that persist in the environment form strong biofilm when AdrA is over expressed (Rahman *et al.*, 2007). This study has also shown an inverse regulation in biofilm formation and motility by GGDEF and EAL domain proteins. It is not known whether GGDEF domain proteins from different bacteria can cross complement other phenotype besides biofilm and motility.

7.3 Paper III

Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium

In S. Typhimurium multiple copies of the GGDEF and EAL domain proteins raised the question that perhaps the chromosomally encoded GGDEF and EAL domain proteins are redundant. Our studies, paper III (Kader *et al.*, 2006) and paper IV (Simm *et al.*, 2007) and the previous studies (Garcia *et al.*, 2004; Romling *et al.*, 2000) showed that they are not redundant. GGDEF and EAL domain proteins have very defined and specific functions. From our previous study we know that GGDEF and EAL domains contribute to the c-di-GMP turnover and transition from sessility to motility (paper I) (Simm *et al.*, 2004), we attempted a systematic approach to dissect the involvement of GGDEF domain proteins in rdar phenotype development.

In S. Typhimurium, analysis of GGDEF domain proteins revealed that five proteins have GGDEF domain and seven proteins shared EAL domain with GGDEF domain (Romling, 2005). Among these twelve proteins, it was known that chromosomal inactivation of STM0385 (AdrA) gives a bdar phenotype which indicates that the expression of cellulose in that mutant has been diminished (Romling *et al.*, 2000; Zogaj *et al.*, 2001). The role of CsgD as a transcriptional activator for AdrA is known. The regulation of curli by CsgD has been delineated. But the contribution of the other 11 GGDEF domain containing proteins was not known. An investigation of GGDEF domain in phenotypes was carried out in S. Typhimurium (Garcia *et al.*, 2004). It was shown that a subset of the proteins are involved in expressing biofilm in complex medium and another GGDEF domain protein STM1987, is involved in biofilm regulation in nutrient deficient medium (Garcia *et al.*, 2004; Lasa, 2006). In our studies, we have shown the hierarchical involvement of all the GGDEF domain proteins in rdar morphotype development. We have also investigated the regulatory network and the contribution of c-di-GMP (Kader *et al.*, 2006).

It was shown earlier that a point mutation in the CsgD promoter can help the strain to produce biofilm at 37ºC (Romling *et al.*, 1998b; Romling *et al.*, 2000). We have over-expressed AdrA in UMR1 and shown that the whole rdar morphotype is up-regulated. When YhjH is up-regulated, the whole rdar morphotype is down regulated. It indicates that not only cellulose is a target for c-di-GMP but the whole rdar phenotype is under the control of c-di-GMP. This finding is an indication that curli is also regulated by c-di-GMP. In the next phase, we have checked the amount of the structural protein (CsgA) of curli fimbriae. The experimental data showed that CsgA is also up-regulated in a *bcsA* mutant when AdrA is up-regulated. The level of CsgA is almost diminished when YhjH is up-regulated. These findings established that curli is a target for c-di-GMP.

It was shown that high levels of c-di-GMP through over-expression of diguanylate cylcase AdrA enhance levels of mRNA for CsgD. The analysis of the protein level for CsgD also showed that it is up-regulated. This means that the regulation of CsgD by c-di-GMP takes place on mRNA level of transcriptional stability. It has also shown that opposing effects can occur when there is lower level of c-di-GMP which was modulated by over-expressing the EAL domain protein YhjH. To investigate whether there is any posttranscriptional regulation of CsgD by c-di-GMP, we have over-expressed CsgD with YhjH in a *csgD* mutant. It was observed that the level of CsgD protein is going down which indicates that CsgD is regulated on a post transcriptional level.

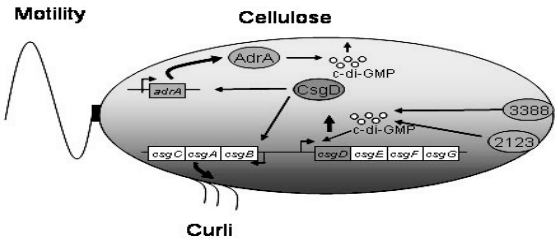

Figure 6. Simplified model for the regulation and task distribution of GGDEF domain proteins in S. Typhimurium UMR1 (Gerstel *et al.*, 2003; Kader *et al.*, 2006; Romling *et al.*, 1998b; Romling *et al.*, 2000; Simm *et al.*, 2004). GGDEF domain proteins contribute to the different pools of c-di-GMP. STM2123 and STM3388 are the two important GGDEF domain proteins which contribute to the rdar phenotype as well as CsgD level (Kader *et al.*, 2006; Simm *et al.*, 2007).

We have investigated the specific functions of GGDEF domain proteins in rdar morphotype development. Among the chromosomal inactivation of all the GGDEF domain proteins, STM3388 and STM2123 give rise to a down regulated rdar phenotype. The double knock out with AdrA and a triple knock out of *adrA*, STM2123 and STM3388 has shown a diminished rdar phenotype. The temporal and spatial regulation of rdar morphotype development was studied. The molecular regulatory analysis showed that the level of CsgD is going down in the STM3388 and STM2123 mutant as well as the respective double and triple mutant with AdrA. The down-regulation of structural gene for curli was also observed in these mutants.

The level of c-di-GMP was tested for the triple mutants of *adrA*, STM2123 and STM3388 with further refined analysis by HPLC and MS. The developed technique has allowed us to detect the level of c-di-GMP in wild type S. Typhimurium and the isogenic GGDEF domain mutants. It was shown that the level of c-di-GMP concentration has been varied with time and STM2123 as well as STM 3388 do not significantly contribute to the total c-di-GMP pool. On the other hand, AdrA is required for the majority of the intracellular c-di-GMP produced at 16h but not extensively involved in c-di-GMP production at 10 and 24 h. Since CsgD is not

affected in *adrA* mutant but AdrA is contributing to the intracellular c-di-GMP level, this observation indicates that there might be pools of c-di-GMP. It is known now that there are multiple c-di-GMP binding proteins which have the PilZ domain (Amikam and Galperin, 2006; Romling and Amikam, 2006). It is now known that c-di-GMP binds with the receptor of c-di-GMP *in vivo* (Christen *et al.*, 2007; Lee *et al.*, 2007; Pratt *et al.*, 2007; Ryjenkov *et al.*, 2006).

Temperature regulation of S. Typhimurium UMR1 in rdar phenotype was shown previously to be over-come by a promoter-up mutation in CsgD (Romling *et al.*, 1998b). At higher temperature S. Typhimurium can not express CsgD and CsgA, so none of the biofilm matrixes are expressed. On the other hand, promoter up mutation of CsgD leads to the expression of CsgD and CsgA. We have investigated whether higher level of c-di-GMP can aid the bacteria to express CsgD at higher temperature. We have shown that CsgD is expressed and biofilm is formed at higher temperature when AdrA is over expressed.

This study has shown the task distribution of GGDEF domain proteins and the molecular regulation rdar morphotype development. It has established CsgD expression and curli are new targets for c-di-GMP. It has been shown that there is a positive feed back loop for c-di-GMP to control CsgD. The over-coming effect of temperature regulation of biofilm formation has been delineated. It would be interesting to know the mechanism of action of CsgD to play a central role in regulating the multiple processes.

7.4 Paper IV

Role of EAL-containing proteins in multicellular behaviour of *Salmonella enterica* serovar Typhimurium

As paper III (Kader *et al.*, 2006) describes the hierarchical involvement of GGDEF domain proteins in rdar morphotype development, we have studied the role of EAL domain proteins in rdar morphotype in this study extending former studies (Kader *et al.*, 2006; Romling *et al.*, 2000; Simm *et al.*, 2004). We have investigated different phenotypes regulated by EAL domain proteins. The phenotypes include pellicle formation, formation of biofilm in liquid culture, plate assessment for rdar phenotype and the flagella mediated motility.

The chromosomal inactivation of the EAL domain proteins showed that a subset of the EAL domain proteins have up-regulated rdar phenotype in plate assessment. Among the total 14 GGDEF domain proteins STM1703 (YciR), STM4264, STM3611 (YhjH) and STM1827 showed effect on biofilm matrix regulation or phenotypic expression. Quite recently the biochemical analysis of some of the proteins has been uncovered. STM1703 which has both GGDEF and EAL domain has been shown to have phosphodiesterase activity (Weber *et al.*, 2006). Chromosomal inactivation of STM1703 and STM4264 has shown an up-regulated rdar morphotype. The domain structure of STM4264 includes EAL domain and does not have any GGDEF domain. The rdar phenotype was slightly up-regulated in STM3611 and STM1827 mutant. Molecular analysis of the master regulator CsgD revealed that with the up-regulation of rdar phenotype, level of CsgD and c-di-GMP has been up-regulated. The level of CsgD and c-di-GMP has been highly up-regulated in STM1703 and STM4264 mutant. Over-expression of STM1703 has down-regulated CsgD and c-di-GMP. Analysis of the protein YciR in *E. coli* through microarray and chromosomal inactivation showed that the *csgBAC* operon is up-

regulated in a *yciR* mutant (Weber *et al.*, 2006). These findings are in agreement with the findings that curli is regulated by c-di-GMP (Kader *et al.*, 2006).

The biofilm formation in this study covers the pellicle formation (biofilm formed in liquid air interface), clumping and adherence to the glass surface. In our screen many of the EAL domain proteins did not show any effect on biofilm formation. Overall analysis of the biofilm attributes of the mutants indicates that STM1703 (YciR) formed the most significant amount of biofilm at 48 h at 28ºC in liquid culture. The other two mutants STM4264 and STM1827 showed a moderate increase. The pellicle formation was increased in STM1703 and STM4264 mutant and moderately up-regulated by STM1827. The STM3375 (YhdA or CsrD in *E. coli*) which is known to regulate the carbon storage system in *E. coli* (Jonas *et al.*, 2006) was up-regulating the biofilm. The STM3375 homologue in *E. coli* has shown recently that they do not have any diguanyl cyclase or phosphodiesterase activity (Suzuki *et al.*, 2006). The prediction of the function of CsrD indicates that the protein might bind small RNAs and expose them for degradation by RNAseE.

In *V. cholerae*, c-di-GMP positively regulates biofilm formation and negatively regulates virulence gene expression. Consequently EAL domain proteins play an important role in the transition of smooth and rugose phenotype (Beyhan *et al.*, 2006; Rashid *et al.*, 2003). It has been proposed that c-di-GMP plays a crucial role in the transition from persistence in the environment to survival in the host. In S. Typhimurium, STM1703 showed the PDE activity but STM1703 has both GGDEF and EAL domain. In *V. cholerae,* an infection induced gene *cdpA* encodes a GGDEF and EAL domain protein. It is known to have a PDE activity due to the inactive GGDEF domain. CdpA has been shown to inhibit biofilm formation (Tamayo *et al.*, 2008). But it did not show any effect in colonization of the infant mouse small intestine. C-di-GMP is known to have effects in early infection and colonization. The higher level of c-di-GMP results in reduced colonization and also the reduction of ToxT transcription (Tamayo *et al.*, 2008).

The screening of the EAL domain proteins showed that STM1344 gives rise to completely opposing effect on the biochemical activity (Romling and Amikam, 2006). The STM1344 is an unconventional EAL domain protein which was also screened by signature/tagged transposon mutagenesis with differential recovery from wild type and immunodeficient mice (Hisert *et al.*, 2005). STM1344 was named *cdgR* (c-diguanylate regulator) and is required to resist host phagocyte oxidase *in vivo*. Chromosomal inactivation of *cdgR* decreased bacterial resistance to hydrogen peroxide and decreased killing to bacterial macrophages (Hisert *et al.*, 2005). These findings show c-di-GMP to regulate host-pathogen interaction involving antioxidant defense and cytotoxicity.

It was shown that besides EAL domain proteins HD-GYP domain is involved in degradation of c-di-GMP. Mutational inactivation of H and D in conserved HD-GYP domain resulted in the loss of activity in *X. campestris* (Fouhy *et al.*, 2006; Ryan *et al.*, 2006). This domain is highly conserved in free-living bacteria, plant and animal pathogens, beneficial symbionts and organisms associated with environmental niches (Ryan *et al.*, 2006). The synthesis of virulence factors and dispersal of biofilm are regulated by HD-GYP domain as the EAL domain is regulating the virulence factor and host pathogen interaction (Hisert *et al.*, 2005; Tamayo *et al.*, 2008; Tischler and Camilli, 2005). The presence of two kinds of domains to degrade c-di-GMP is not known yet.

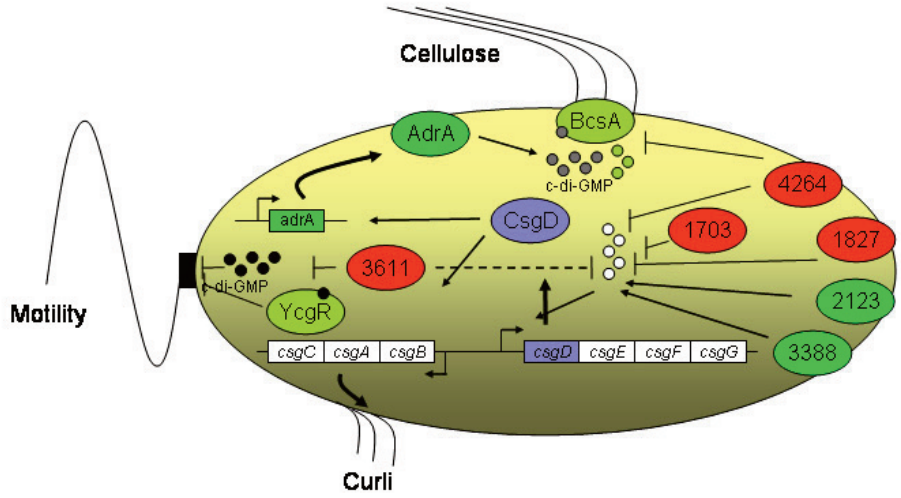

Figure 7. Illustration of task distribution of GGDEF-EAL domain proteins in rdar morphotype development. Among the GGDEF domain proteins STM2123 and STM3388 contribute to the rdar phenotype development (Kader *et al.*, 2006). EAL domain proteins STM4264, STM1703 and STM1827 contribute to the rdar phenotype development (Simm *et al.*, 2007). STM3611 regulates the motility (Girgis *et al.*, 2007; Ko and Park, 2000; Simm *et al.*, 2004) . C-di-GMP binds with PilZ domain proteins YcgR and BcsA which in turn contributes to motility and biofilm phenotypes (Amikam and Galperin, 2006; Romling and Amikam, 2006; Ryjenkov *et al.*, 2006).

The overall analysis of our results and the findings from other investigators (Bobrov *et al.*, 2005; Kulasakara *et al.*, 2006; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005; Tischler *et al.*, 2002) has placed EAL domain protein in a very important position that regulates multiple processes within the cells. We have started to get detailed understanding of the biochemical properties of the domain. Further analysis of the functions of this domain *in vivo* will add new information to our understanding.

7.5 Manuscript

Polynucleotide phosphorylase promotes biofiolm formation and motility through differential regulation of EAL domain proteins in *Salmonella enterica* serovar Typhimurium

Bacterial biofilm formation has acquired increased attention since biofilm is involved in multiple positive and detrimental effect. The mechanism of action and detailed regulatory network is extenssively studied to get an insight into the full biological benefit of bacterial communities. Extensive investigation has been performed to understand the functions and

regulation of polynucleotide phosphorylase. It is known that PNPase is involed in virulence gene expression (Clements *et al.*, 2002). Domain architechture of PNPase showed that it has an S1 domain. Inactivation of the S1 domain leads to a trauncated form of PNPase which has residual PNPase activity. PNPase is present in all forms of life and involved in autoregulation.

In S. Typhimurium the detailed regulatory network for the regulation of biofilm formation is known. Th structure and function for PNPase was studied to a greater extent. The role of GGDEF and EAL domain proteins, apart from the classical gene regulatory proteins, were nailed down. In this study, we have investigated the phorolytic exoribonuclease PNPase which is evolutionary conserved and participates in degradation and modification of RNA. This study connects the regulatory network of c-di-GMP with PNPase regulation.

Mutational inactivation of PNPase was found to be negatively affecting the biofilm formation associated with multicellular bahaviour development and motility in S. Typhimurium. The plate assessment of CR binding showed that rdar phenotype is down-regulated in *S.* Typhimurium compared to PNPase mutant. The down-regulation of cellulose was also investigated by calcoflour binding assay and the mutant showed down-regulated rdar phenotype. The molecular analysis showed that the master regulator for biofilm formation, CsgD was also down-regulated in the PNPase mutant compared to the wild type S. Typhimurium MC1. The structural gene for curli expression was down-regulated in the PNPase mutant. Analysis of mRNA for CsgD and quantitative reverse transcriptase PCR (qRT-PCR) analysis showed that the mRNA of CsgD is less expressed in the mutant than in the wild type. The transcriptional analysis of CsgA revealed that CsgA protein is down-regulated in the mutant compared to the wild type transcriptional level. These data suggest that inactivation of the PNPase has a significant impact on biofilm formation.

Since c-di-GMP is up-regulating the biofilm formation through GGDEF domain and down-regulated by EAL domain (Simm *et al.*, 2004), we have over-expressed AdrA and YhjH in a PNPase mutant. It has been shown that the rdar morphotype and the master regulator CsgD are up-regulated in AdrA over-expressed PNPase mutant. CsgD is down-regulated in YhjH over-expression background. The c-di-GMP has also up-regulated the curli subunit CsgA. On the other hand, the full length inactivated PNPase is complemented by c-di-GMP for rdar phenotype expression. We have investigated the impact of c-di-GMP on the protein level of PNPase expression. We could consistently show that the level of PNPase protein was down-regulated when AdrA is over-expressed and slight up-regulated when YhjH is over-expressed.

In previous screening of EAL domain proteins in rdar phenotype development, it was found that two proteins are involved extensively in rdar phenotype up-regulation (Simm *et al.*, 2007). Inactivation of STM1703 (YciR) and STM4264 lead to an up-regulated phenotype and showed the rdar phenotype even at 37ºC. It was also shown that the regulation of motility is dominated by the EAL domain protein YhjH (Girgis *et al.*, 2007; Ko and Park, 2000; Rahman *et al.*, 2007; Simm *et al.*, 2004). Analysis of available microarray data showed that two proteins STM4264 and STM3611 (YhjH) are affected significantly among these 19 GGDEF and EAL domain proteins in PNPase mutant background. The inactivation of PNPase and STM4264 showed that the rdar morphotype and the level of CsgD recovered in the double mutant.

The PNPase mutant showed reduced motility. We have shown that with the aid of YhjH, PNPase mutant can move as the wild type. Chromosomal inactivation of YhjH showed down-

regulation of motility. Further more, the double knock out of YhjH and PNPase showed that the bacteria is almost paralysed.

In *Y. pestis* optimal functioning of the type III secretion system (T3SS) in cell culture infection assays requires PNPase (Rosenzweig *et al.*, 2007). The normal activity of the T3SS can be regained expressing S1 domain. The PNPase is involved in multiple regulation including the virulence gene expression (Clements *et al.*, 2002; Ygberg *et al.*, 2006). C-di-GMP is appreciated as a second messenger molecule and involved in regulation of biofilm and virulence gene expression (Kader *et al.*, 2006; Ryan *et al.*, 2007; Simm *et al.*, 2004; Tischler and Camilli, 2004, 2005). This study showed an interconnection between the c-di-GMP signalling and PNPase regulation through the differentail regulation of EAL domain proteins. The future direction will focus on the interconnection of c-di-GMP signalling and PNPase regulation of virulence gene expression.

7.6 Concluding remarks and future perspectives

GGDEF and EAL domain proteins in large numbers of bacteria, enhance interest to study the common and different attributes of bacteria in signal transduction. It has been shown that GGDEF and EAL domain proteins are involved in c-di-GMP synthesis and degradation (Simm *et al.*, 2004). Some of the biological functions of c-di-GMP mainly related to biofilm formation and virulence are now known. A handful of the targets and phenotypes affected by c-di-GMP have been explored. The c-di-GMP signalling network is very complex. Since the biofilm matrix components are under the control of c-di-GMP which is modulated by GGDEF and EAL domain proteins in different groups of bacteria, the discovery can lead to understanding the underlying mechanisms when designing anti-biofilm treatments.

GGDEF and EAL domain proteins were studied in model organism like S. Typhimurium, *E. coli*, *V. cholerae*, *P. aeruginosa* and *X. campestris* to nail down the functions of these proteins. In S. Typhimurium, the roles of GGDEF-EAL domain proteins were studied in rdar phenotype development and biofilm formation. In *V. cholerae*, the role of c-di-GMP was shown in virulence gene expression while an inventory of the EAL domain proteins was made in *P. aeruginosa*. The HD-GYP domain which is involved in c-di-GMP degradation was characterized in *X. campestris*. HD-GYP domain containing protein regulates the virulence gene expression and environmental adaptation (Ryan *et al.*, 2007). Future studies will add new phenotypes which are under the control of c-di-GMP. The targeted proteins affected by high pool of c-di-GMP can be identified by whole genomic or proteomic approaches.

It is evident that GGDEF and EAL domain proteins are not redundant in the chromosome while they have specific task distribution in multicellular morphotype development. The molecular regulation, effectors mechanism and biochemical analysis have been discovered to some extend. It is claimed that c-di-GMP can be produced in localized micro-compartments. There might be pools of c-di-GMP within the cells. Single bacterial cell contains multiple c-di-GMP binding receptors which could contribute to localized c-di-GMP pools. Experimental evidence is necessary to establish this hypothesis.

In S. Typhimurium, GGDEF and EAL domain proteins regulate the biofilm matrix components cellulose and curli through the master regulator CsgD. The regulation of CsgD by c-di-GMP occurred at both the transcription and post transcriptional level. C-di-GMP is regulating other phenotypes besides biofilm. C-di-GMP is assumed to be involved in multiple

processes. A link between the function and regulation of c-di-GMP and poly nucleotide phosphorylase has been established. It was found that there is a differential regulation of EAL domain proteins in PNPase. The contribution of different RNAses in rdar morphotype regulation could be delineated together with PNPase.

Besides the role of c-di-GMP in bacteria, there were attempts to investigate the applications of the molecule c-di-GMP. It was shown in *S. aureus* that treatment of bacteria with external c-di-GMP can reduce the biofilm (Karaolis *et al.*, 2005b). There is a great need of mucosal adjuvant in vaccinology since discovery of new adjuvant has not been a very successful process. It was shown that c-di-GMP is an immunostimulatory molecule (Karaolis *et al.*, 2007). It is known that c-di-GMP can inhibit the proliferation of certain cancer cell (Karaolis *et al.*, 2005a; Steinberger *et al.*, 1999).

Since c-di-GMP is playing a crucial role in transition from planktonic state to biofilm state, it will be interesting to know the effect of c-di-GMP in the transition from environment to causing infection. Some of the studies are shedding light already on this direction. Most of the effect of c-di-GMP and the functions of the GGDEF and EAL domain proteins are shown under laboratory conditions. So the environmental growth and persistence due to the elevated level of c-di-GMP will be an interesting direction to study.

Related publications not included in the thesis

I. Römling, U., Kader, A., Divariam, D., Simm, R., and Kronvall, G. (2005) Worldwide distribution of clone C strains in the aquatic environment and cystic fibrosis patients. *Environ. Microbiol.* 7: 1029-38

II. Rahman, R., Simm, R., Kader, A., Basseres, E., Römling, U., and Möllby, R. (2007) The role of c-di-GMP signalling in an *Aeromonas veronii* biovar *sobria* strain. *FEMS Microbiol. Lett* 273: 172-179

III. Jonas, K., Tomenious, H., Kader, A., Normark, S., Römling, U., Belova, L. M., and Melefors, Ö. (2007) Roles of curli, cellulose and BapA in *Salmonella* biofilm morphology studied by Atomic force microscopy. BMC Microbiol. 7:70 doi:10.1186/1471-2180/7/70

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